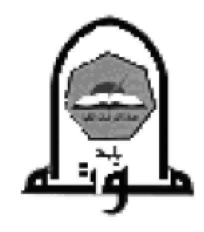


Mu’tah University

Deanship of Graduate Studies

Determination of the Activity and Kinetics of Proteases from Different Plant Sources

By

Fatamh Nasser Alomari

Supervised by

Dr. Omar Atrooz.

A Thesis Submitted to the Deanship of Graduate Studies
in Partial Fulfillments of the Requirements for the Degree of
Master of Biology - Department of Biology

Mu'tah University, 2013

Dedication

To
My father
My mother
My brother Majid
My friend Ibtesam

Fatamh N. Alomari

Acknowledgment

Great thanks to ALLAH who guided me during my life and still supporting me with his mercy and driving.

My special thanks and appreciation to my supervisor Dr. Omar Atrooz for his continuous advice and support throughout this work.

I would like to express my deep thanks to all my colleagues and all the entire staff members of the Biology Department, Mutah University, for helping, providing a good working environment, working assistance whenever necessary, and for sharing their scientific knowledge.

Fatamh N. Alomari

Table of Contents

List of Figures

List of Table

List of Appendices

Abstract

Determination of the Activity and Kinetics of Proteases from Different Plant Sources

Fatamh Nasser Alomari

Mu'tah University, 2013

Our result illustrate that the crude enzyme extracts of *Mentha piperita* , *Rosmarinus officinalis, Thymus capitatus* and *Olea europaea* have a proteolytic activity within optimum pH (3-7) and optimum temperature (35 - 45 ^{o}C) . Study of the ionizable groups in or around active site of these proteases by Dixon-Webb's plot reveals the occurrences or the presence of aspartic acid, glutamic acid and cysteine amino acids in or around the active site , interestingly , these proteases may be belonged to the acid proteases and neutral proteases. Enzymatic kinetics studies(k_m, v_{max}, v_{max} / k_m) indicated that casein is the most specific substrate for proteases of *R. officinalis* followed by *M. piperita, T. capitatus* and *O. europaea,* while egg albumin is the best substrate of *M. piperita* followed by *T. capitatus, O. europaea.* In contrast, results of k_m values indicated that casein has high affinity for proteases of *R. officinalis*, *T. capitatus*, *M. piperita* and *O. europaea*

الملخص

تحديد نشاطية وحركية أنزيم البروتييز من مصادر نباتية مختلفة

فاطمة ناصر العميري

جامعة مؤته، 2013

تشير نتائج الدراسة إن مستخلص الإنزيم من نباتات النعنع والحصلبان والزعتر وورق الزيتون لها نشاطية لتحطيم البروتين عند درجة حموضة (3 الى 7) ودرجـة حرارة مثلى عند (35 الى 45) ، وعند دراسة المجموعات المتأينة حـول الموقـع النشط للأنـزيم مـن خـلال Dixon-Webb's plot وجـد إن aspartic acid glutamic acid and cysteine موجودة في او حول الموقع النشط للأنزيم وهـذه الأنزيمات تنتمي للإنزيمات الحمضية والقاعدية ، الدراسات الحركية للأنزيمات (k_m, v_{max}, v_{max} / k_m) وجد ان الكازين أفضل مادة متفاعلة مع الإنزيم في نبات الحصلبان ثم يليها نبات النعنع ثم الزعتر واخيرا ورق الزيتون ووجد ان بياض البيض أفضل مادة متفاعلة مع الإنزيم في نبات النعنع ثم نبات الزعتر ثم ورق الزيتون واثبتـت دراسـة km ان الكازين له فعالية كبيرة في نبات الحصلبان ثم الزعتر ثم النعنع واخيـرا ورق الزيتون .

Chapter One
Introduction

1.1 Introduction

The requirements of many industrial applications of proteases are very different from their physiological properties. Very few proteases from naturally occurring organisms without any modification are used in industrial processes. There are many extensive researches carried out on microbial and plant proteases (major resource) to obtain and improve the industrial and new biotechnological applications (Vanndita *et al*., 2013).

Proteases are industrial enzymes that catalyze the hydrolytic reactions by cleaving peptide bonds in protein (Akhtaruzzaman *et al.*, 2012). They are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood-clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase-activating cascade) (Ibrahim *et al.*, 2012b). They may be classified as two major groups; exopeptidases and endopeptidases. They are also classified into five major groups according to the character of their catalytic active site and conditions of action (aspartate, cysteine, threonine, metallo and serine proteases) (Al-sherhi and Mostafa, 2004). Alternatively, proteases may be classified by the optimal pH in which they are active into three types (acid proteases, neutral proteases and basic proteases (or alkaline proteases)).(Sandhya et al., 2004)

Proteases are involved in numerous physiological processes that include food digestion, cell maintenance, cell signalling, wound healing, cell differentiation and cell growth (Vanndita *et al*., 2013). Any defect in the control of protease activity leads to undesired and unregulated proteolysis. This is the cause of many diseases, such as Alzheimer's disease, stroke, cancer, viral infections and cataracts (Powers *et al*., 2002), thus inhibitors of proteases have the potential to provide successful therapeutics for a wide range of diseases (Rawlings *et al*., 2006).

The proteases are characterized according to the basis of biochemical properties like optimum pH, optimum temperature, molecular weight, N-terminal sequence, isoelectric point, critical amino acid required for the catalytic function, and substrate specificity.

Most proteases require metal ions for their maximum activity, for active configuration and for enzyme stabilization. Metal ions like Ca^{+2}, Ba^{+2}, Fe^{+3}, Mg^{+2} and Mn^{+2} are known to play a major role in enzyme stabilization by increasing the activity and thermal stability of enzymes at higher temperatures (Lee *et al*., 2003; Kumar 2001; Kotlova *et al*., 2007; Johnvesly; Rattray *et al*., 1995 and Naik 2001).

However, Li *et al*., (2007) have reported that some heavy metals have inhibitory effects to majority of proteases such as Hg^{+2}, Co^{+2}, Cd^{+2}, Ni^{+2}, Cu^{+2}, Ag^{+2}, Fe^{+2} and Zn^{+2} (Moallaei *et al*., 2006).

In general, proteases have different characteristics and broad substrate specificity and hydrolyze variety of natural as well as synthetic substrates. Natural substrates include keratin, gelatin, casein, albumin collagen and elastin are hydrolyzed by specific types of alkaline proteases such as collagenase, elastase, keratinase and insect cuticle degrading proteases. Proteases also has the capacity to hydrolyze many synthetic and chromogenic substrates.

Synthetic substrates such as succinyl-Ala-Ala-Ala-p-nitroanilide, succinyl-Ala-Pro-Ala-p-nitroanilide, succinyl-Ala-Ala-Pro-Leu-p-nitroanilide and glutaryl-Ala-Ala-Pro-Leu-p-nitroanilide are specific substrates for elastase enzyme, while N- Suc-Ala-Ala-Pro-Phe-p-nitroanilide is specific substrate for chymotrypsin and N-benzoyl- Phe-Val-Arg-p-nitroanilide for trypsin like proteases (Rozs,2001).

In the current study, the studied plants: *Mentha piperita*, *Olea europaea* , *Rosmarinus officinalis* and *Thymus capitatus* are used widely in folk medicine and literature review survey indicate that there are no studies or researches about their proteases activity or characteristics. However, the main purposes of this study were to use green leaves of the above plants as a sources to extract the proteases enzymes and to study their optimum pH , temperature, kinetics and their specific activity.

1.2 Aims of the study :

1. To search for more sources of proteases from plants.
2. To characterize the protease enzymes from *Mentha piperita* (Mentha), *Olea europaea* (Olive), *Rosmarinus officinalis* (Rosemary) and *Thymus capitatus* (Thyme) .
3. To determine and compare the activity and kinetics of the proteases from *Mentha piperita* (Mentha) , *Olea europaea* (Olive), *Rosmarinus officinalis* (Rosemary) and *Thymus capitatus* (Thyme).

Chapter Two
Literature Review

2.1 Enzyme characteristics:

Proteolytic enzymes catalyzing the hydrolytic cleavage of specific peptide bonds in target proteins are called as proteases. These proteolytic enzymes are widely distributed in nearly all plants, animals and microorganisms (Christeller, 2005; Joanitti *et al.,* 2006).

Proteases form a large group of enzymes and found in a wide variety of sources (animals, plants, fungi and bacteria) (Joanitti *et al*., 2006; Christeller, 2005; Haq *et al*., 2004; Lawrence and Koundal, 2002; Ryan, 1990). They are molecules of relatively small size and are compact, spherical structures that catalyze the peptide bond cleavage in proteins (Polgar, 1989) and perform nutritional and regulatory role in nature.

Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale (Chouyyok *et al*., 2005).

Proteases are generally used in detergents (Barindra *et al*., 2006), food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Rao *et al*., 1998; Paranthaman *et al*., 2009). In general, they play a key role in a variety of biological processes, both at the physiological level and in infection (Paranthaman *et al*., 2009).

Proteases are classified as serine, threonine, cysteine, aspartate and glutamic acid proteases. Many pathological disorders are caused by deficiencies in the normally exquisite regulation of the activity of proteolytic enzymes, resulting in abnormal tissue destruction and the aberrant processing of other proteins.

2.2 Importance

Proteases have a first place in the world market of enzymes, estimated at ~US$3 billion (Leary *et al*., 2009), since they play an important role in biotechnology, their proteolysis changes the chemical, physical, biological, and immunological properties of proteins. Hydrolysis of food proteins, for example, is carried out for various reasons: improvement of the nutritional characteristics, retarding deterioration, modification of different functional properties (solubility, foaming, coagulation, and emulsifying capacities), prevention of undesired interactions, change of flavors and odors, and removal of toxic or inhibitory factors (Pardo *et al*., 2000).

2.2.1 Medical and Biological Applications

In general, Proteases play key roles in many medical and biological processes. The proteolytic events catalyzed by these enzymes serve as mediators of signal initiation, transmission and termination in many of the cellular events such as inflammation, apoptosis, blood clotting and hormone processing pathways (Ivanov *et al.,* 2006).

Plants have the capacity to synthesize certain biologically active substances, which play a major role in plant defense, against insect pests and microbial attacks. Some of these include defense proteins like proteinase inhibitors (PIs), amylase inhibitors, lectins and class of pathogenesis-related proteins (Ryan, 1990; Tatyana *et al.,* 1998; Padul *et al.,* 2012). These proteins are specifically produced in the plant upon biotic stresses and protect the plant tissue from the damage (Ryan, 1990; Tatyana *et al.,* 1998).

Proteases also have an important application in the pharmaceutical industry. Plant extracts with a high content of protease enzymes have been used in traditional medicine for a long time. They have been used for the treatment of cancer (Batkin *et al*., 1988; Targoni *et al*., 1999), as antitumorals (Guimaraes-Ferreira *et al*., 2007; Otsuki *et al*., 2010), for digestion disorders (Kelly, 1996; Mello *et al*., 2008), and swelling and immune-modulation problems (Leipner *et al*., 2001; Lotti, 1993; Melis, 1990; Otsuki *et al*., 2010). A good example is bromelain, derived from pineapple, which has been shown to be capable of preventing edema, platelet aggregation and metastasis due to it's capacity of modifying cell surface structures by peptide cleavage.

Salas *et al*., (2005) reviewed the pharmacological activity of plant cysteine proteases, emphasizing their role in mammalian wound healing, immunomodulation, digestive conditions, and neoplastic alterations.

2.2.2 Industrial and Biotechnological Applications

Proteases play a great role in many industrial processes (Gupta, Beg, & Lorenz, 2002), they are used in pharmaceuticals (Sjodahl *et al*., 2002), medical diagnosis, decomposition of gelatin on X-ray films, in textiles (Tunga, Shrivastava, & Banerjee, 2003), Milk-clotting and food industry as well as in some industrial applications such as detergent, food (calf rennet, in cheese making) (Smith *et al*., 1989), leather, food complement (Kleef *et al*., 2004; La Valle *et al*., 2000), preparation of soy sauce (Wang & Wang, 2004), production of emulsifiers (Pardo *et al*., 2000), isolation of genetic material (Genelhu *et al*., 1998), meat tenderization (Cheng *et al*., 2010) and in studying the structure of proteins and peptides, (Li, D *et al*., 1997).

Proteases occur naturally in all organisms. Although most proteases are derived from microbial origin (Tang *et al*., 2008) such as bacteria, fungi and yeast, Plant

proteases with important industrial applications have also been characterized (Fahmy et all, 2004). Furthermore, commercial use of proteases with different origins was reported (Capiralla *et al*., 2002).

Proteases play central role in the biochemical mechanism of germination (Bewley and Black, 1994) and are responsible for breakdown of storage proteins (Rao, 1998) .

These enzymes are important in a number of diverse and crucial biological processes; for example, they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogenicity, and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao *et al*., 1998). Protease inhibitors are proposed to regulate the proteolytic activity in a wide variety of physiological and pathological processes in vivo (Monard, 1988; Solomon *et al*., 1996;Turk *et al*. 1997).

Proteases are used in the degumming of silk goods, in the manufacture of liquid glue, in the preparation of cosmetics, in the preparation of detergents, in the meat tenderization, in the preparation of cheese, in medicine preparation and in agriculture as growth promoters (Katunumaet al., 2003).

Measuring hydrolytic activity on synthetic substrates is a simple way to know the cleavage specificity of these enzymes, which provides important information for biotechnological applications, as with the production of bioactive peptides from food proteins (Silva and Malcata, 2005).

2.3 Classification

There are currently five known classes of proteases: serine, threonine, cysteine, aspartic acid, glutamic acid and metallo- proteases. The most widely recognised system of enzyme classification is according to the proteases catalytic mechanism, structure (clans and families) and individual protease (Powers, 2002) .

The Catalytic mechanism classifies proteases according to catalytic centre composition (Schaller, 2004). The two different fundamental catalytic mechanisms for hydrolysis are: For serine, threonine and cysteine proteases, the key catalytic nucleophile is an intrinsic component of the active site (the residue being Ser, Thr or Cys respectively), while aspartic acid, glutamic acid and metallo- proteases use an activated water molecule as the nucleophile (Mykles, 2001 and Demuth, 1990**)**.

Structurally, they are classified according to clans and families. Clans are defined as groups of proteases that have arisen from a single origin and are based on the similarity of tertiary structures, with each clan representing an homologous set of proteases. Clans may be composed of more than one class of protease as illustrated by the clan PA which contains examples of both serine and cysteine proteases. Within each clan, families are identified by a letter that represents the

type of hydrolysis it catalyses followed by a unique number (1, 2, 3…)(Table 1)(Rawlings *et al*, 2006) .

According to the individual classification, proteases are defined to be individual and distinct if specificity and sensitivity to inhibitors is different, they are of different catalytic types or are encoded by different genes within the same organism (Turk, 1999).

Table (1)

Serine Protease Clans[a]

Clan	Members	Example	Catalytic residues	Distribution
		"Classic" Catalytic Triad Serine Proteases		
PA(S)	301	chymotrypsin	His-Asp-Ser	B, Ar, F, Pl, An, V
SB	91	subtilisin	Asp-His-Ser	B, Ar, Pr, F,Pl,An,V
SC	64	carboxypeptidase	Y Ser-Asp-His	B, Ar, Pr, F, Pl, An,
SK	14	Clp protease	Ser-His-Asp	B, Ar, Pr, F, Pl,An,V
		"Novel" Serine Proteases		
SE	16	D-Ala-D-Ala carboxypeptidase A	Ser-Lys	B, Ar, Pl, An
SF	24	signal peptidase I	Ser-Lys/His	B, Ar, F, Pl, An, V
SH	7	cytomegalovirus assemblin	His-Ser-His	V
SM	7	C-terminal processing protease-1	Ser-Lys	B, Ar, Pl
SN	4	dipeptidase E	Ser-His-Glu	B, An
PB(S)	4	penicillin amidohydrolase precursor	N-terminal Ser	B, Ar, Pr

a The MEROPS database classifies proteases based on structural. Catalytic residues are listed in the order that they appear in the primary sequence. B, bacteria; Ar, archaea; Az, archezoa; Pr, protozoa; F, fungi; Pl, plants; An, animals; V, viruses. In addition to the proteases listed below, 21 serine proteases have been identified that are not yet assigned to a clan.(Rawlings *et al*, 2006)

Proteases are broadly divided into either exopeptidases or endopeptidases depending on their site of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as exopeptidases. If the enzyme cleave peptide bonds distant from the termini of a substrate, they are classified as endopeptidases. (Turk, 1999).

However, with the advent of molecular biology, proteases are also grouped into families based on the following; chemical nature of the catalytic or active sites, mechanism(s) of action, and the evolutionary relationship of their three-dimensional structure (Beynon *et al*., 1989 and Rao *et al*., 1998).

Based on statistically significant similarities in sequence and structure of proteases, Rawlings *et al.,* (2006) have devised a classification scheme and terms this database MEROPS. MEROPS divides proteases into clans based on catalytic mechanism and families on the basis of common ancestry (Table 1) (Mykles, 2001). At present, over 48,000 peptidase gene sequences have been classified into 50 clans and 184 families. The catalytic triad is located in the active site of the enzyme, where catalysis occurs and is preserved in all serine protease enzymes. The triad is a coordinated structure consisting of three essential amino acids: histidine (His 57), serine (Ser 195) and aspartic acid (Asp 102). Located very close to one another near the heart of the enzyme, these three key amino acids play an essential role in the cleaving ability of the proteases. The whole reaction is summarized in figure 1.

The proteolytic events catalysed by these enzymes serve as mediators of signal initiation, transmission and termination in many of the cellular events such as inflammation, apoptosis, blood clotting and hormone processing pathways (Ivanov *et al*., 2006). The activities of these enzymes need to be strictly regulated and controlled because at higher concentrations may be potentially damaging the cells and organism (Rawlings *et al*., 2004).

The MEROPS Database (Rawlings *et al*., 2010) considers seven families of proteases: Aspartic, Cysteine, Glutamic, Metallo, Asparagine, Serine and Threonine. In plants, five classes of endoproteases have been described: Serine, Cysteine, Aspartic, Metallo and Threonine (Rawlings *et al*., 2006).

In 1995, Seemuller (1995) classified proteinases as hydrolyses (Group 3) and within the Subgroup 4, i.e. those which are able to hydrolyze peptide bonds. They have been divided into six mechanistic classes including cysteine proteinases, serine proteinases, aspartic proteinases, metaloproteinases, threonine proteinases and unknown type proteinases.

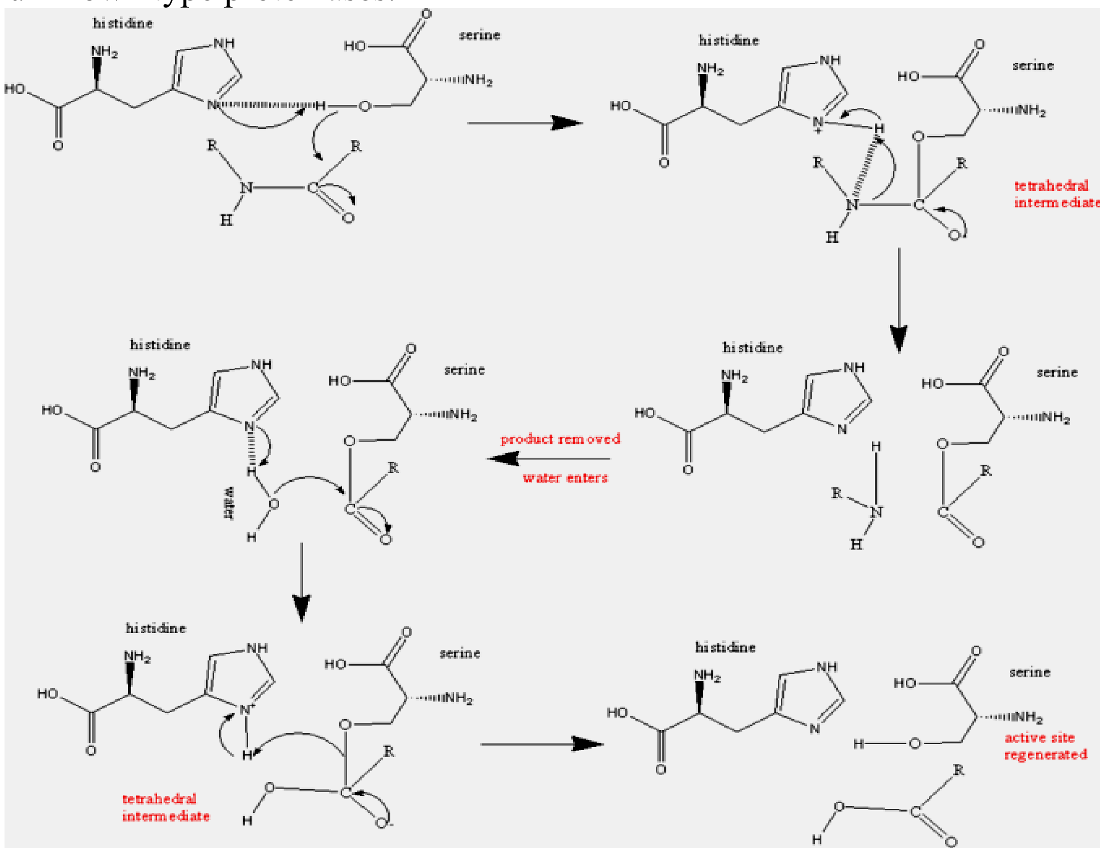


Figure (1)

Reaction mechanism of serine protease

(source:http://en.wikipedia.org/wiki/Serine protease)

(Rawlings et al, 2006

2.4 Bacterial and Fungal Proteases

Most of bacterial proteases that have been purified are extracellular metalloproteases. These enzymes are all between approximately 20 – 60 kDa (Liao *et al*., 1998, McKevitt *et al*., 1989, and Sexton *et al*., 1994).

Bacteria has a number of diverse proteases that have been purified and characterized from multiple strains (Ogino *et al.*, 1999; Olson *et al*., 1992; Kessler *et al*.,1998). Interestingly, these proteases are all extracellular and directly associated with species virulence (act as an exotoxins). However, Bacteria secrete proteases as preproenzyme, which is then processed into its mature form; for example, *Pseudomonas aeruginosa* secretes an elastase as a preproenzyme (MW 53 kDa), which is then processed into its mature form (MW 33 kDa) (Kessler el al, 1998). It is a neutral metalloprotease, requiring zinc for enzymatic reactivity and calcium for stability (Powers, 2002).

Fungi also secretes a wide variety of proteases than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al.*, 2006). Several species of strains including fungi (*Aspergillus flavus, Aspergillus melleu,* ….etc) and bacteria (*Bacillus licheniformis, Bacillus firmus,* ….etc) are reported to produce proteases (Haq *et al.*, 2006).

The pH and incubation temperature have an effect on the production of proteases. However, some microorganisms produce heat stable proteases which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature (Al-Shehri, 2004; Haq *et al.*, 2006).

2.5 Animal Proteases

Proteases digestion is the process which helps an organism to convert macromolecules into monomers that can be absorbed through the epithelium and therefore, nutritionally useful to that organism. It has been established that fishes and higher vertebrates utilize the same enzymes and hormones in the breakdown of proteins into amino acids (Smith, 1989). In fish, the specific levels of digestive enzyme activities are dependent on age, diet, season, and/or ambient temperature (Munilla-Morán, 1996).

The most three serine proteases that have been studied in detail are chymotrypsin, trypsin, and elastase. They are synthesized by the pancreaticacinar cells, secreted in the small intestine and are responsible for catalyzing the hydrolysis of peptide bonds. They are similar in structure from their X-ray structures. The differing aspect lies in the peptide bond that is being cleaved (scissile bond). Each of these digestive serine proteases target different regions of a

polypeptide chain based upon the side chains of the amino acid residues surrounding the site of cleavage.

2.6 Plant Proteases

The degradation of proteins is important for plant development. In several key stages of plant development such as during germination of seeds and during leaf senescence, storage and other proteins are degraded to release amino acids for transport to the most actively growing regions of the plants. The activity of these proteases is low in the youngest leaves and very high in old senescing leaves (Haq *et al.*, 2006).

Ichishima, 1991 have reported that most of the plant proteases are neutral or alkaline and there are few acid proteases; in general, they are widely distributed in the plant seeds and play important physiological roles in the metabolism of seed proteins.

Plant proteases play a role in plant cell adaptation to environmental conditions, selectively degraded oxidized proteins (produced by oxidative stress) and are involved in many aspects of plant physiology and development (van der Hoorn, 2008), protein turnover, degradation of misfolded proteins, senescence and the ubiquitin/proteasome pathway (Beers *et al.*, 2000), responsible for the post-translational modification of proteins (Schaller, 2004), diversity of cellular processes, including photoinhibition in the chloroplast, defense mechanisms, programmed cell death, and photomorphogenesis in the developing seedling (Leary *et al*., 2009). Proteases are thus involved in all aspects of the plant life cycle ranging from movilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller, 2004).

Proteases have been identified and studied from the latex of several plant families such as Asteraceae, Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae and Euphorbiaceae (Domsalla and Melzig, 2008).

Most plant-derived proteases have been classified as cysteine proteases and more rarely as aspartic proteases (Rawlings *et al*., 2006).

Proteolytic enzymes derived from plants are very attractive since they can be active over a wide range of temperature and pH (Uhlig, 1998). Enzyme preparations from plant extracts have been used in industrial processes for a long time. Although, the great majority of commercial enzymes have been obtained mainly from microbial sources but plant enzymes are becoming increasingly important, with applications in industrial processes, biotechnology and pharmacology.

The current study was investigate the protease activity in four plants that are used widely in folk medicine :*Mentha piperita*, *Olea europaea* , *Rosmarinus officinalis* and *Thymus capitatus*.

Mentha piperita (Mentha) is a perennial glabrous and strongly scented herb belonging to family Lamiaceae. The plant is aromatic, stimulant and used for allaying nausea, headache and vomiting. Mentha has an astringent, antiseptic, antipyretic, antispasmodic, anticatarrhal, antimicrobial, rubefacient, stimulant, emmenagogue and anti-aging properties due to the biologically active constituents and is widely used against upset stomachs, inhibits the growth of certain bacteria, and can help soothe and relax muscles when inhaled or applied to the skin (Shasany *et al*., 2000). It was reported that *M. piperita* extract exhibit a proteolytic activity against *T. cinnabarinus* (Ren *et al*., 2009).

Olea europaea (Olive) has been used as a folk remedy for combating fevers and other diseases such as malaria (Zarzuelo,1991), cardiovascular benefits, decreased blood pressure in animals and increased blood flow in coronary arteries, some hypoglycemic activity, relieved arrhythmia, prevented intestinal muscle spasms , and possesses antioxidant activity. (Samova, 2004), effective against human immunodeficiency virus (HIV) (Lee-Huang *et al*., 2003).

Rosmarinus officinalis (Rosemary) is a common aromatic evergreen shrub grown in many parts of the world. The fresh and dried leaves are used frequently as a food preservative, a flavoring agent, rich in vitamins, rich source of minerals (http://www.nutrition-and-you.com/rosemary-herb.html).

Rosemary has been used to treat renal colic, dysmenorrhea, stimulate hair growth, used in aromatherapy to treat anxiety-related conditions and to increase alertness (Dorman,*et al*., 2003).

Rosemary leave extract contains antioxidant compounds (caffeic acid and rosmarinic acid). Therefore, it plays a potential role in cancer, hepatotoxicity, and inflammatory therapies (Khorshidi, *et al*., 2009). Paris, 1993 reported that the carnosolic acid extracted from *Rosmarinus officinalis* had activity against human immunodeficiency virus.

Thymus capitatus (Thyme) is a genus containing about 350 species belongs to the family Lamiaceae. Thyme leaves have been used in foods for the flavour, aroma and preservation for meat, fish and food products (Gruenwald *et al*., 2004). It possesses biological properties: antimicrobial activity , antifungal activity, antiseptic and antioxidant activity (Schaller, 2004), and as antioxidant activity (Rasooli *et al*., 2006). Thyme is an excellent source of iron, manganese, calcium and vitamin K (Sasaki *et al*., 2005).

The literature survey of *Mentha piperita, Olea europaea* , *Rosmarinus officinalis* and *Thymus capitatus* indicated that there is no real study about their proteases activities. This study may be the first to investigate the proteolytic activity of proteases, optimum pH, optimum temperature and the Kinetics in these selected plants.

Chapter Three
Materials and Methods

3.1 Plant Material

Four plants (*Mentha piperita* (Mentha), *Olea europaea* (Olive), *Rosmarinus officinalis* (Rosemary) and *Thymus capitatus* (Thyme)) (Table 2) were freshly collected from local markets. The plants parts were thoroughly washed with distilled water and refrigerated at 4^{o}C until used.

Table (2)

The botanical data of the four selected plant species used in this study.

#	Scientific name	Common name (English)	Common name (Arabic)	Family	Parts used
1	*Mentha piperita*	Mentha	النعناع	Lamiaceae	Leaves
2	*Olea europaea*	Olive	الزيتون	Oleaceae	Leaves
3	*Rosmarinus officinalis*	Rosemary	الحصلبان ، إكليل الجبل	Lamiaceae	Leaves
4	*Thymus capitatus*	Thyme	الزعتر	Lamiaceae	Leaves

3.2 Crude Enzyme Extraction

Forty grams (40g) of plant leaves were ground by electric homogenizer. The homogenates were finely powdered in a pre-chilled mortar and pestiles mixed with chilled 0.1 M phosphate buffer at pH 7.0. The homogenized mixture was then filtered and centrifuged at 5000 rpm for 10 minutes. The collected supernatant was used for the estimation of prortien concentration and protein activity (Akhtaruzzaman *et al.*, 2012).

3.3 Protein measurement

Protein concentration was determined by the method of Lowry *et al.*, 1951 using bovine serum albumin (BSA) as standard protein. The amount of the soluble protein was calculated from the standard curve as mg of protein per ml of test samples

3.4 Protease Assay

The protease activity was assayed as described by Fahmy *et al.*, (2004). Briefly, to an eppendorf tube, 50 µl of the crude enzyme source was incubated with 650 µl

of 100 mM sodium acetate buffer pH 4.5, and 100 µl of 1% substrate (albumin, casein and egg albumin) at 37^{o}C for one hour. The reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid. The precipitated proteins were removed by centrifugation at 10,000 rpm and the absorbance of the supernatant was measured at 366 nm. The activity of protease was defined as the amount of enzyme that hydrolyzes 1µmol of amino acids (in terms of tyrosine) from casein per minute under the standard assay conditions.

3.5 pH and Temperature-dependent Studies

A pH dependent assay of the enzyme was conducted using 50 mM acetate buffer pH 3.0-5.0 and 50 mM phosphate buffer pH 6.0-9.0 with pH nterval. The temperature-dependent study was performed by determining the enzyme activity at varying temperatures ranging between 20 and 55^{o}C with ^{o}C nterval (Ibrahim *et al.*, 2012a) .

3.6 Substrate Specificity and Kinetic Studies

Substrate specificity was determined as relative proteolytic activity of crude enzyme according to method described by Patil, 2010 using the following substrates; albumin, casein and egg albumin with minor modifications. Kinetic experiments were performed using varying concentrations of substrate (1%-3%) and the initial velocity values obtained were used to determine the K_m , V_{max} from the double reciprocal plot of the data. Furthermore, initial velocity data at varying pH values (3.0-9.0) was used to construct a Dixon-Webb's plot of log of V_{max}/K_m against the corresponding pH values to determine the possible ionizable groups in the active site of the protease.

Chapter Four
Results and Discussion

Protease plays a vital role in physiological as well as in many pathological processes. Arthritis, tumor invasion and metastasis, infections and number of degenerative diseases have been linked with proteolytic enzymes. They have found extensive applications in bioremediation processes (Ustariz, 2004).

The present study investigates the activity and kinetics of protease enzymes obtained from green leaves of different plants: *Mentha piperita*, *Olea europaea* (Olive), *Rosmarinus officinalis* (Rosemary) and *Thymus capitatus* (Thyme). For substrate specific determination, three substrates were used: casein , albumin and egg albumin.

4.1 Estimation of protein concentration

The protein concentrations in crude enzyme extracts were estimated by Lowry method. Table (3) shows the protein concentration in the crude enzyme extracts of the selected plants. From the standard curve, the protein concentration in the extracts was calculated. Highest protein concentration was found in *Mentha piperita* (58.16 µg/ml) followed by *Rosmarinus officinalis* (31.7 µg/ml), *Thymus capitatus* (5.67 µg/ml) and *Olea europaea* (4 µg/ml) .

4.2 Estimation of the optimum pH and the ionizable groups

The pH activity profiles of proteases of *Rosmarinus officinalis*, *Thymus capitatus* , *Olea europaea* , and *Mentha piperita* are shown in figures (2-5), respectively. The pH activity profile of *Rosmarinus officinalis* was active in the pH range of 6.0 to 7.0, with an optimum at pH 7.0 (relative activity 100%) and showed 60% and 95% relative activity at pH 6.0 and 6.5, respectively. In comparison with *Rosmarinus officinalis, Olea europaea* has one peak at pH 6.5 with relative activity 100% (Figure 3).

Table (3)

Protein concentration (µg/ml) in the crude enzyme extracts of the selected plants. Data are expressed as mean± SD, n=3.

No.	Sample	Protein Concentration (µg/ml)
1	*Mentha piperita* (Mentha)	58.16 ±4.5 µg/ml
2	*Rosmarinus officinalis* (Rosemary)	31.7 ±2.4 µg/ml
3	*Thymus capitatus* (Thyme)	5.67 ±1.6 µg/ml
4	*Olea europaea* (Olive)	4 ±0.5 µg/ml

For *Thymus capitatus*, the profile of pH relative activity was ranged between pH 2.0 and 3.5 with maximum relative activity 65% and at pH 6.0 to 8.0 with maximum relative activity 100% (Figure 4). Therefore, *Thymus capitatus* has two optimum pH at 3.0 and 6.5. On the other hand, *Mentha piperita* has two optimum pH at 3.0 and 9.0 with relative activity 100% and 70%, respectively (Figure 5). In general, presence of two optimum pH may be due to the presence of isoenzymes for the particular protease.

In literature it was reported that the optimum pH for most plants is different and it was ranged from 3.0 to 10.0. for example, Ali *et al.*, 2003 reported that *Carum copticum* has two peaks (maximum activity) at pH 3.0 (acidic) and pH 7.0 (neutral) while for *Allium sepa* has one optimum pH at 10.0 (alkaline). In comparison with fungal proteases, the majority of the pH optimum ranged between 8.0 to 12.0 while for bacteria is varied from one strain to another and ranged between 3.0 and 11.0 (Erlacher *et al.*, 2006; Hajji *et al.*, 2007 ; Zibaee and Bandani, 2009;Charles *et al.*, 2008).

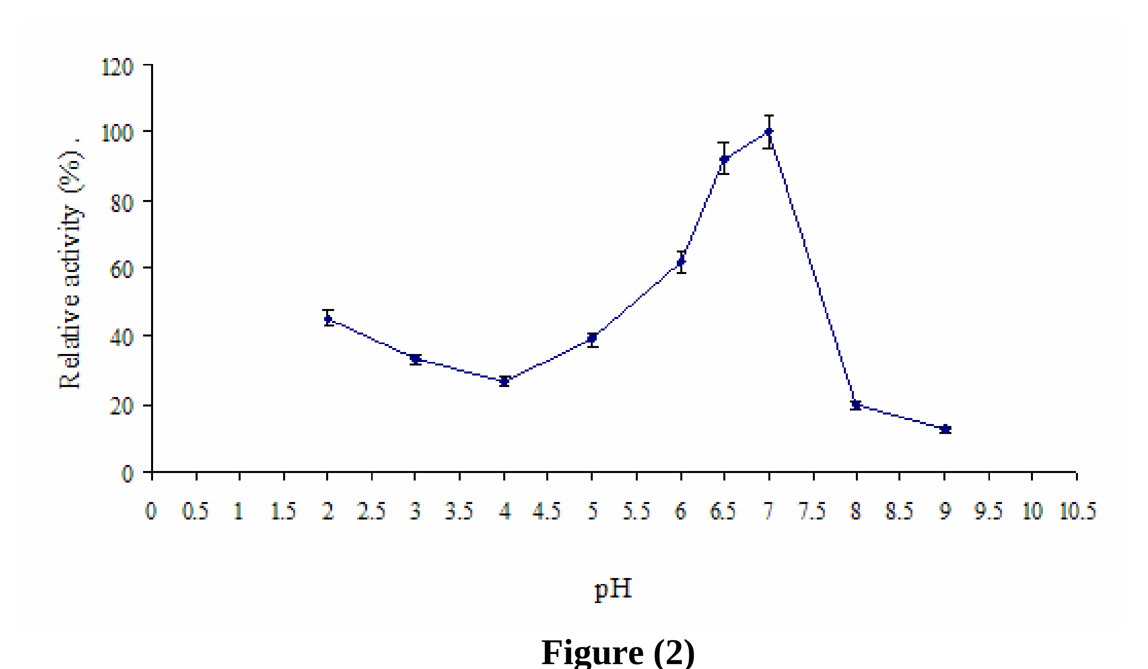


Figure (2)

The relative activity (%) of the pH profile of crude enzyme extract (protease) of *Rosmarinus officinalis.* Data are expressed as mean± SD, n=3.

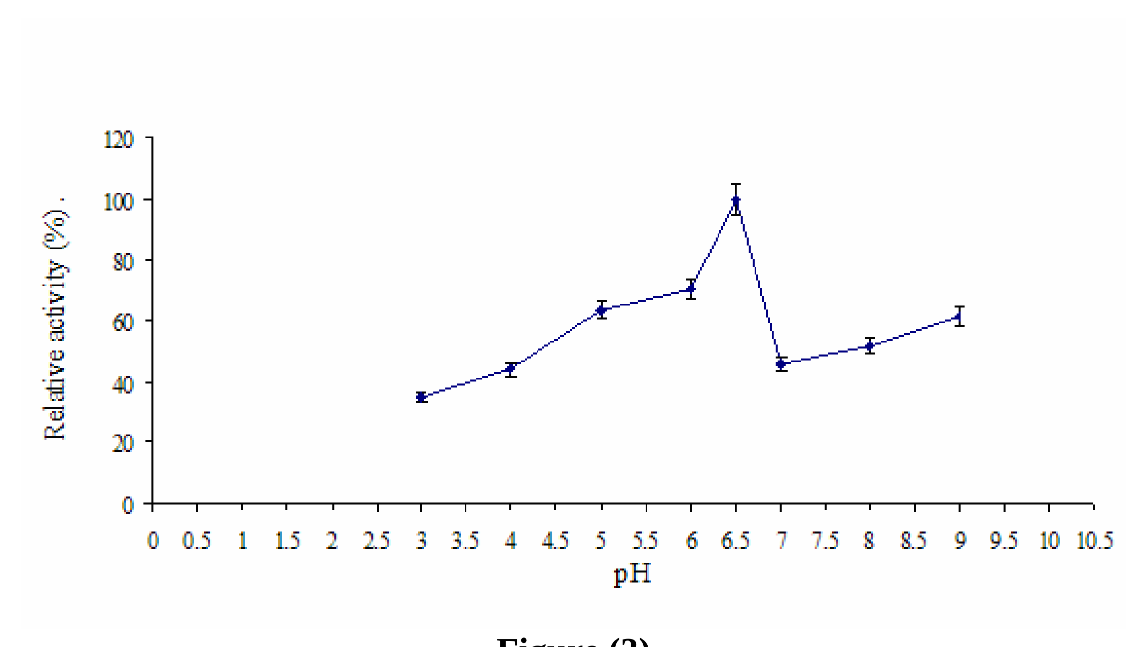


Figure (3)
The relative activity (%) of the pH profile of crude enzyme extract (protease) of *Olea europaea.* Data are expressed as mean± SD, n=3.

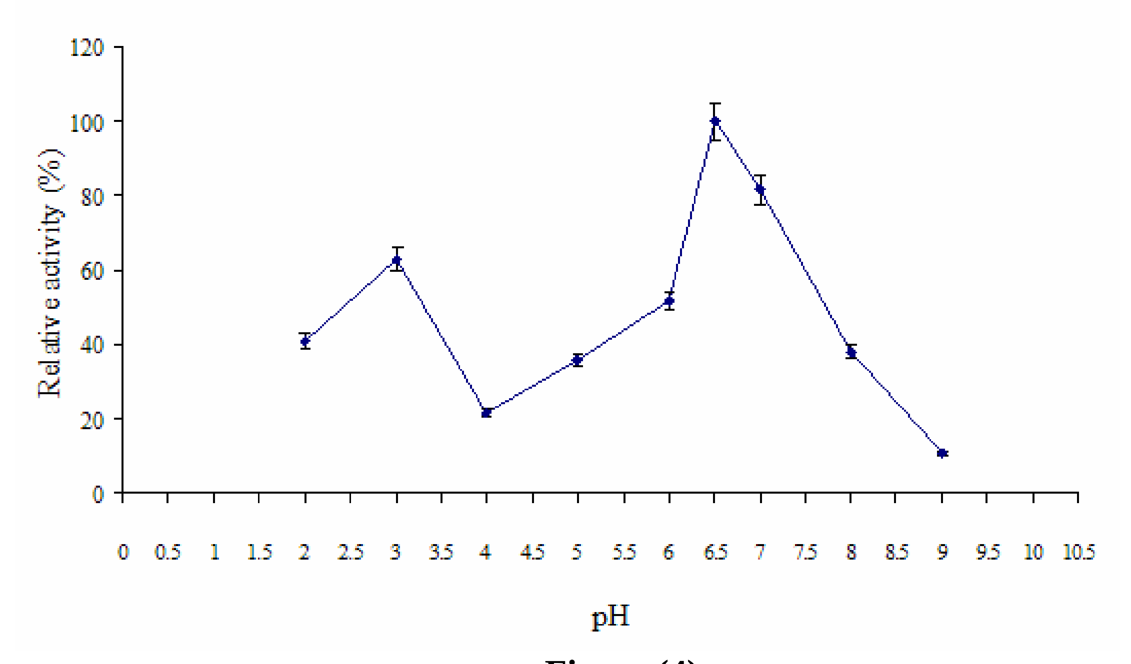


Figure (4)
The relative activity (%) of the pH profile of crude enzyme extract (protease) of *Thymus capitatus* . Data are expressed as mean± SD, n=3.

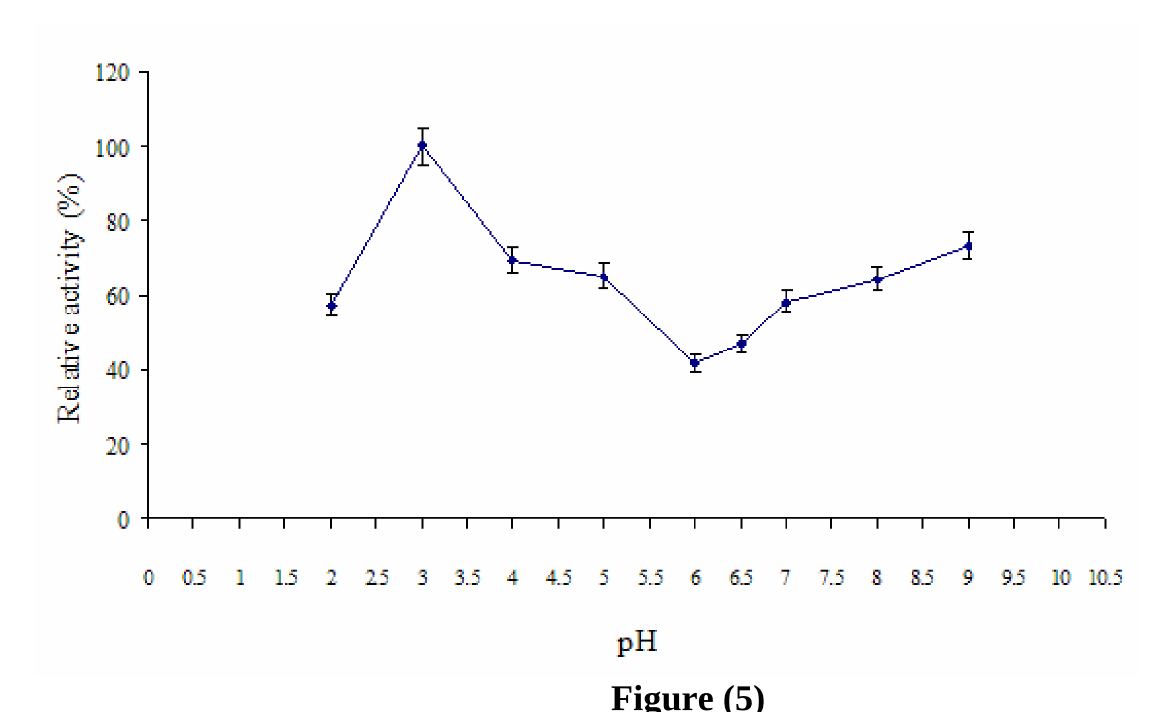


Figure (5)

The relative activity (%) of the pH profile of crude enzyme extract (protease) of *Mentha piperita.* Data are expressed as mean± SD, n=3.

Dixon-Webb's plot was used to determine the ionization groups in the active site of enzymes. According to the Dixon and Webb''protein conformation can be maintained within 4-5 pH units but activity different', it means that the ionizable groups of amino acids in the active site are affected by pH. Analysis of the Dixon-Webb's plot of the *Rosmarinus officinalis*, *Thymus capitatus* , *Olea europaea* , and *Mentha piperita* are shown in figures (6-9), respectively, these figures illustrate the presence of glutamic acid, aspartic acid and cysteine amino acids in most of the selected plants, which indicate that proteases in the crude enzyme extracts of these plants may be belong to the acidic and neutral proteases.

Figure 6, illustrates that *Olea europaea* exhibits an ionizable group with pka1 at pH 4.8 and pka2 at pH 5.2 that indicated the presence of glutamic acid in the active site and therefore belongs to the glutamic acid proteases group. Also, figure 6, illustrates another ionizable group with pka1 at pH 6.8 and pka2 at pH 7.2 that demonstrate the presence of amino acid cysteine in the active site and may be belongs to the group of neutral proteases (Jakubowski, 2013; Voet and Voet, 2004).

In contrast, proteases of *Rosmarinus officinalis* were found two types of proteases groups, the first belongs to the neutral proteases with an ionizable group has pka1 6.3 and 6.7 and the second belongs to the acidic proteases with an acidic ionizable group that has pka1 2.9 and pka2 3.1 may be due to the presence of aspartic acid in and around active site (Figure 7).

In both *Thymus capitatus* and *Mentha piperita* , the ionizable groups were found to exhibit two types, one with pKa1 at pH 6.7 and pKa2 at pH 7.2 (Fig. 8 and 9) which suggests the presence of the amino acid cysteine in the active site of enzyme and therefore may be belong to the neutral proteases group (Cysteine protease), while the other ionizable groups have pKa1 at pH 2.9 and pKa2 at pH 3.2 (Fig. 8 and 9).These results suggest the presence of aspartic acid in and around the active site of these enzymes and may be belong to the aspartic proteases group (Jakubowski, 2013; Voet and Voet, 2004).

Aspartic proteases (EC 3.4.23) are endopeptidases having two aspartic acid residues that are critical for their catalytic activity (Asp32 and Asp215, pepsin numbering) within their active site. Most aspartic proteinases show maximal activity at low pH values (~3-5). They are acidic in nature, having isoelectric points in the range of 3 to 4.5.

Cysteine proteases (thiol proteases) share a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic dyad. They are encountered in fruits and leaves of different plants families and are commonly used in meat tenderizers. They include Actinidain, Bromelain, Calpains, Caspases, Papain and others.

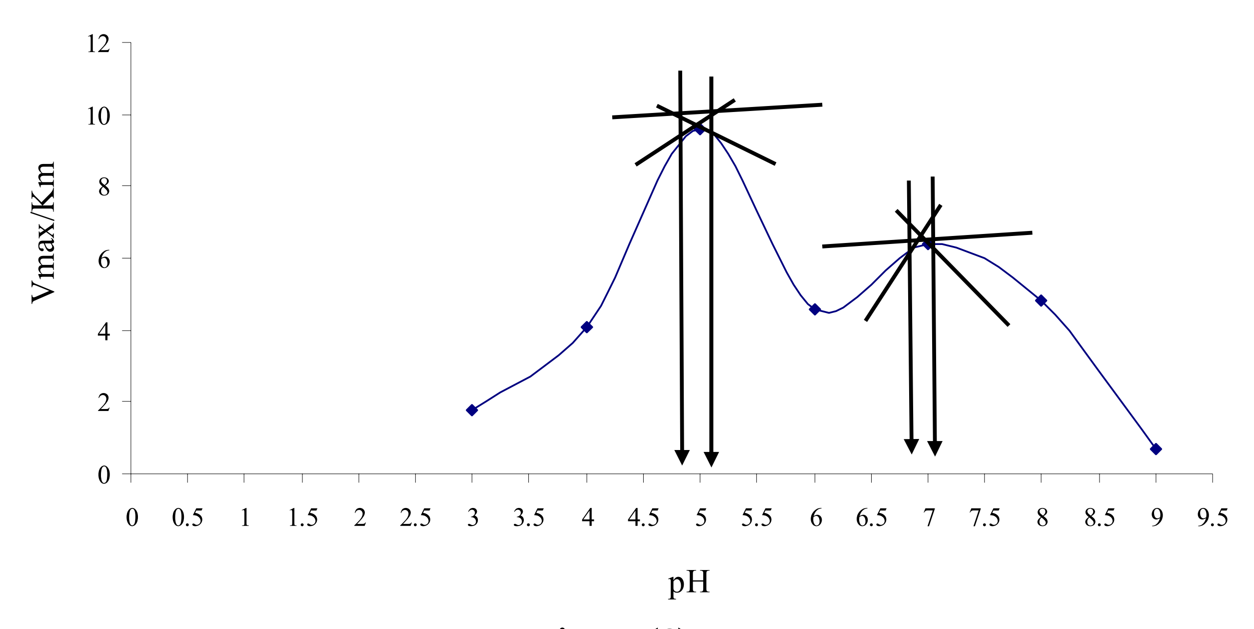


Figure (6)

Determination of possible ionizable groups in the active site of proteases of *Olea europaea* using casein as substrate.

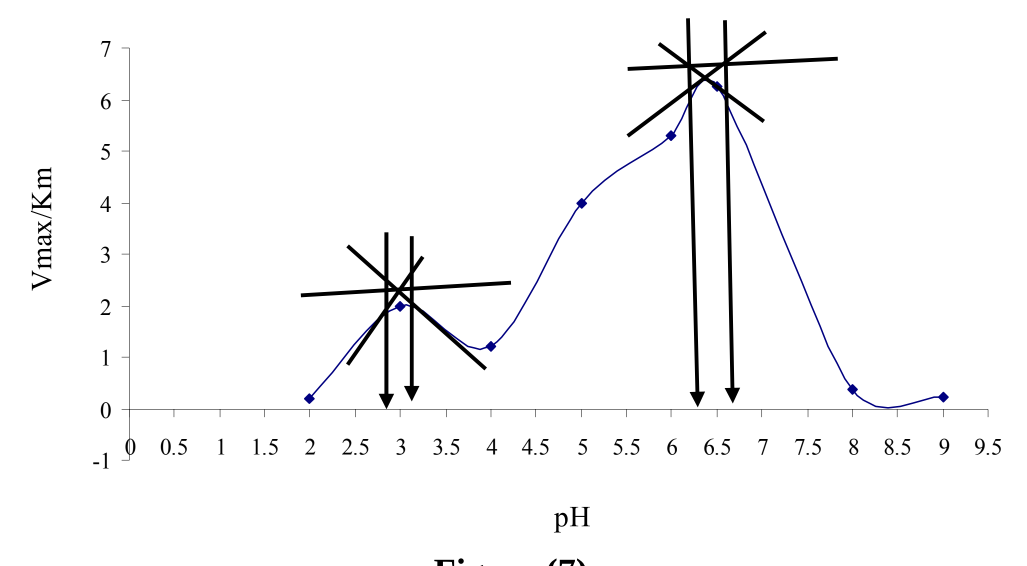


Figure (7)
Determination of possible ionizable groups in the active site of proteases of *Rosmarinus officinalis* using casein as substrate.

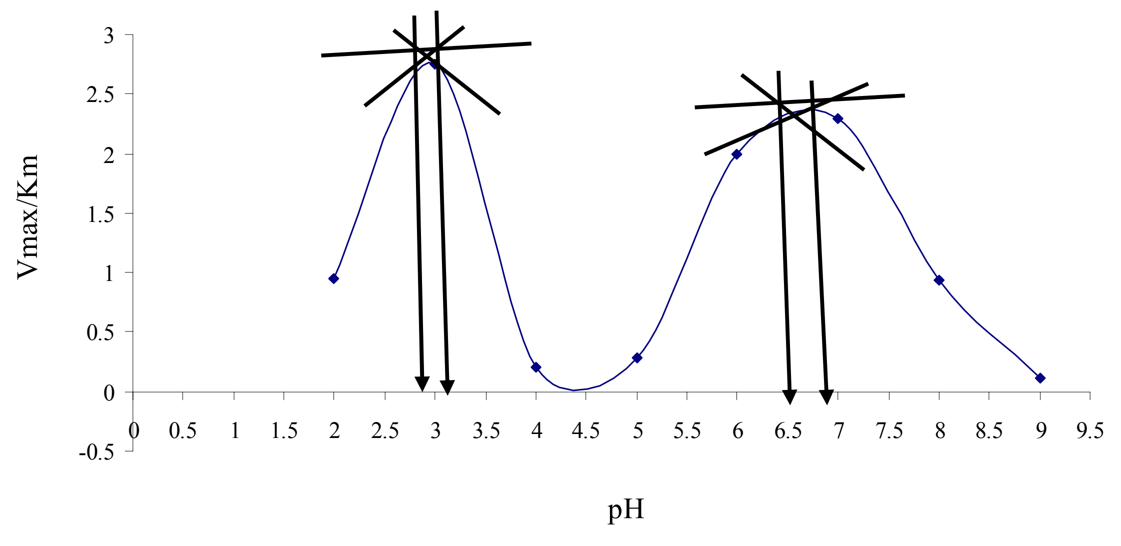


Figure (8)
Determination of possible ionizable groups in the active site of proteases of *Thymus capitatus* using casein as substrate.

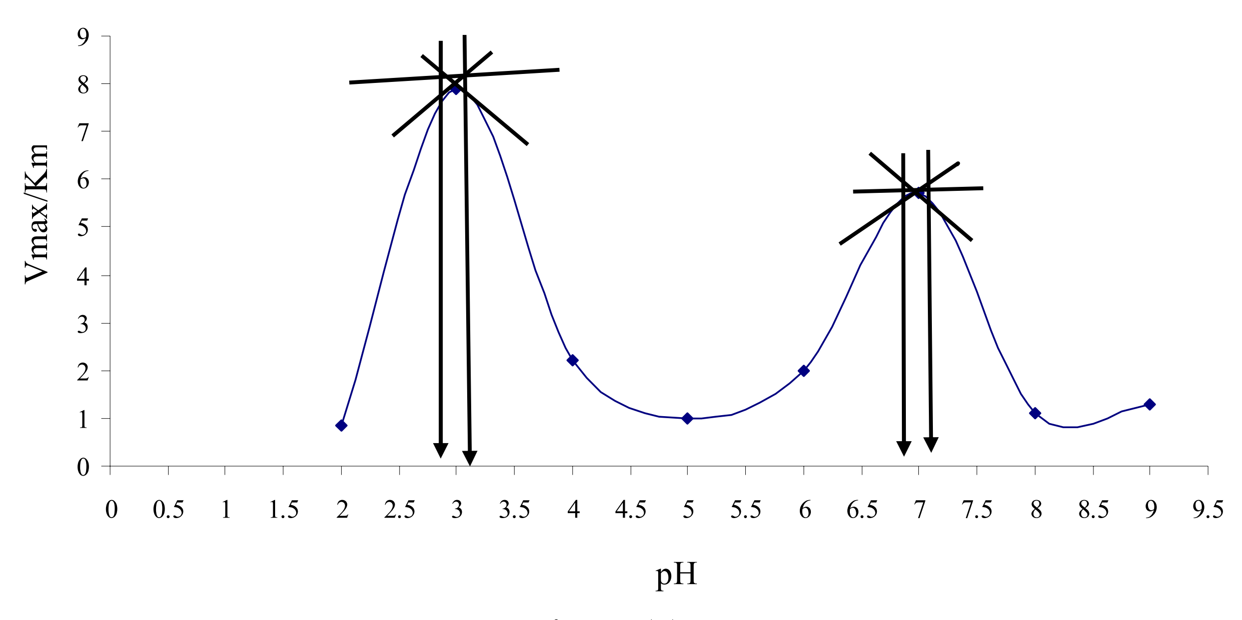


Figure (9)
Determination of possible ionizable groups in the active site of proteases of *Mentha piperita* using casein as substrate.

4.3 Estimation of Optimum Temperature

Optimum temperature is an important factor for industrial enzymes selection because most industrial processes occur at slightly higher than physiological temperature.

Figure (10 and 11) show that maximum relative activity (100%) for proteases of *Rosmarinus officinalis* and *Thymus capitatus* at temperature 40°C. The activity profile of the temperature for *Mentha piperita* was different. The range of the activity from 25 to 60°C with lowest relative activity 60% at temperature 45°C and high activity at temperature 35°C (100%) and relative activity 90% at temperature 50°C (Fig. 12). It was found that the maximum activity of *Olea europaea* proteases was in the temperature regions of 40°C (62%) and 50°C (80%) with a peak at 45°C (100%)(Fig. 13). The thermal stability of proteases may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperatures (Al-Shehri, 2004). The activity profile for elevation temperature above 50°C for all plants show sharply decrease in the activity except for *Olea europaea* that show slightly decrease. The explanation for these results on the protein nature of enzymes that is elevation of temperature above 60°C leads to protein denaturation.

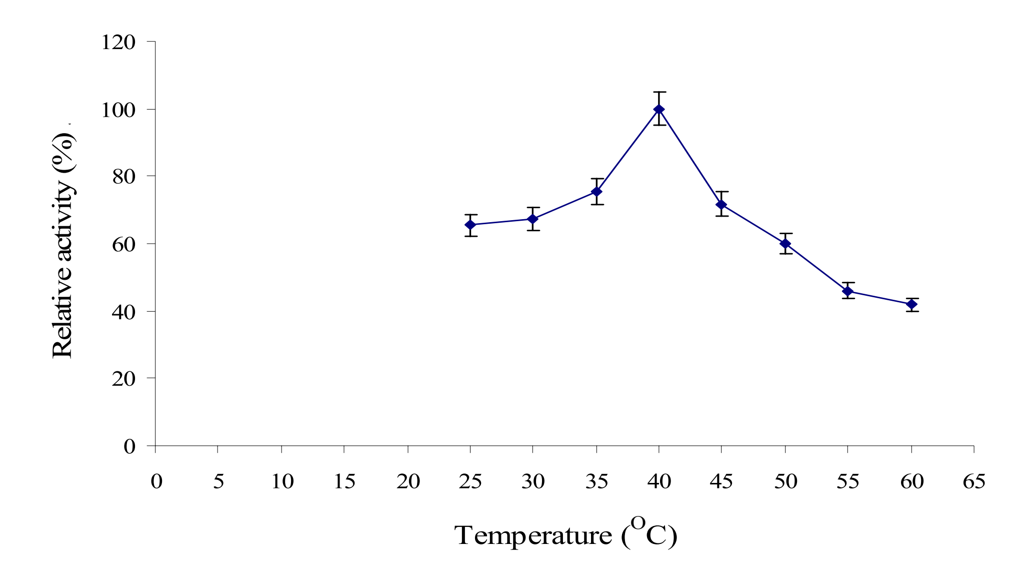


Figure (10)
The relative activity (%) of the temperature profile of crude enzyme extract (proteases) of *Rosmarinus officinalis* . Data are expressed as mean± SD, n=3.

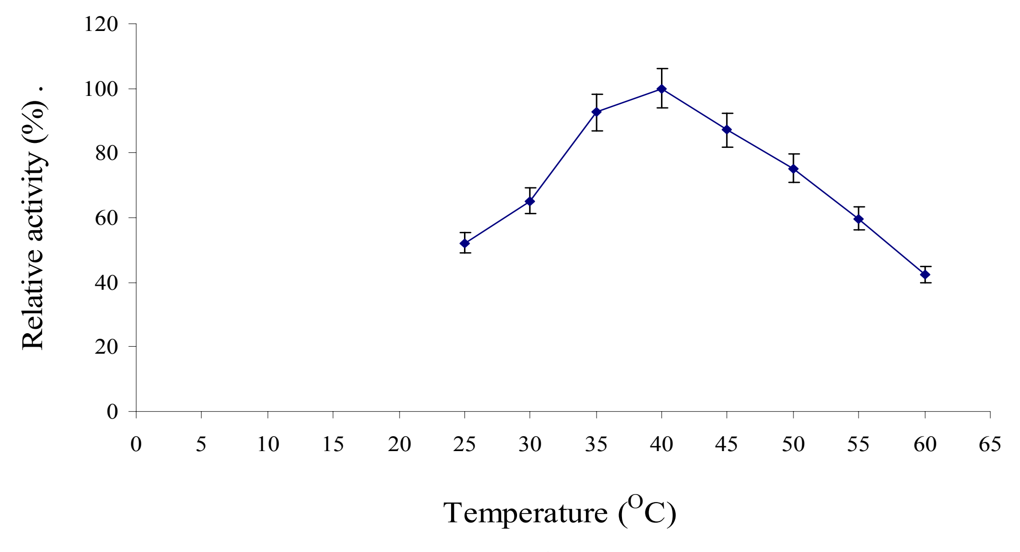


Figure (11)
The relative activity (%) of the temperature profile of crude enzyme extract (proteases) of *Thymus capitatus* . Data are expressed as mean± SD, n=3.

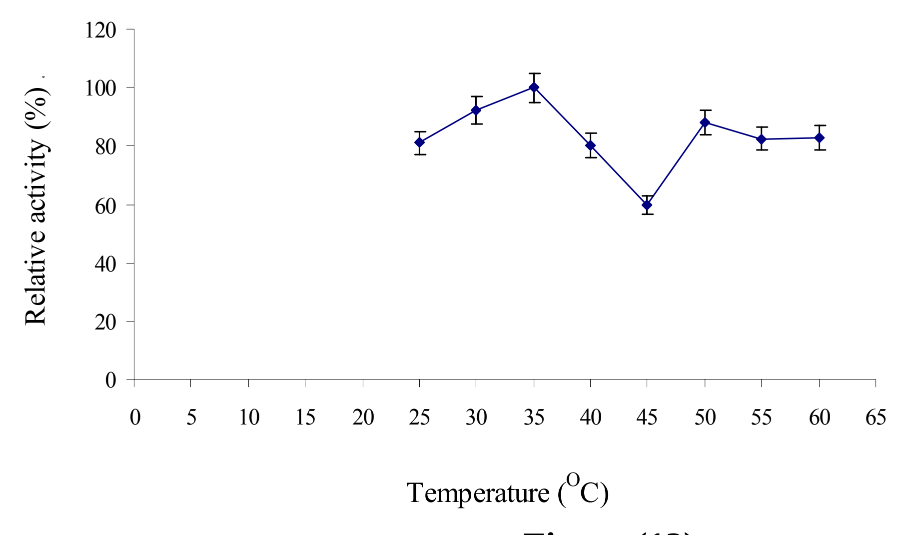


Figure (12)
The relative activity (%) of the temperature profile of crude enzyme extract (proteases) of *Mentha piperita.* Data are expressed as mean± SD, n=3.

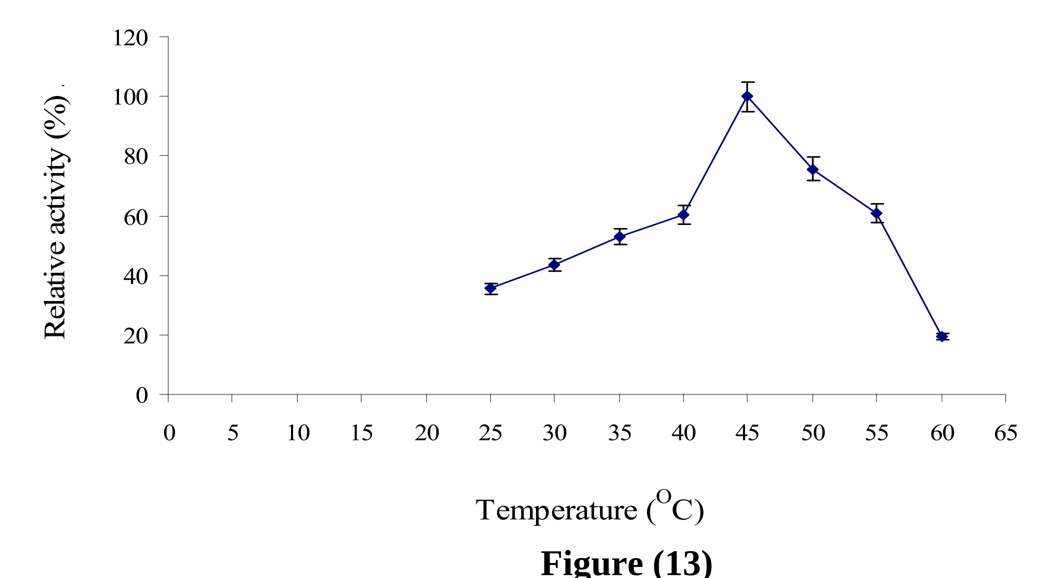


Figure (13)
The relative activity (%) of the temperature profile of crude enzyme extract (proteases) of *Olea europaea.* Data are expressed as mean± SD, n=3.

4.4 Determination of K_m and V_{max}

In addition to pH and temperature, substrate concentrations is consider one of the main factors for determining the activity of enzymes. Also, it can be used for determining the enzyme kinetics (K_m and V_{max}) to illustrate the enzyme affinity and specificity. In the experiments, different concentrations of each substrate of casein, albumin and egg albumin (0.5 %-3.0%) were used. It was found that V_{max} was reached at concentration 3% for the three substrates.

Lineweaver-Burk plots was used to calculate the K_m and V_{max} of proteases enzymes. Figures (14-16) illustrate the plots of K_m and V_{max} for the proteases of the *Rosmarinus officinalis, Mentha piperita, Olea europaea* and *Thymus capitatus* and all the results are summarized in table (4). Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) of the protease enzymes were determined using the above substrates.

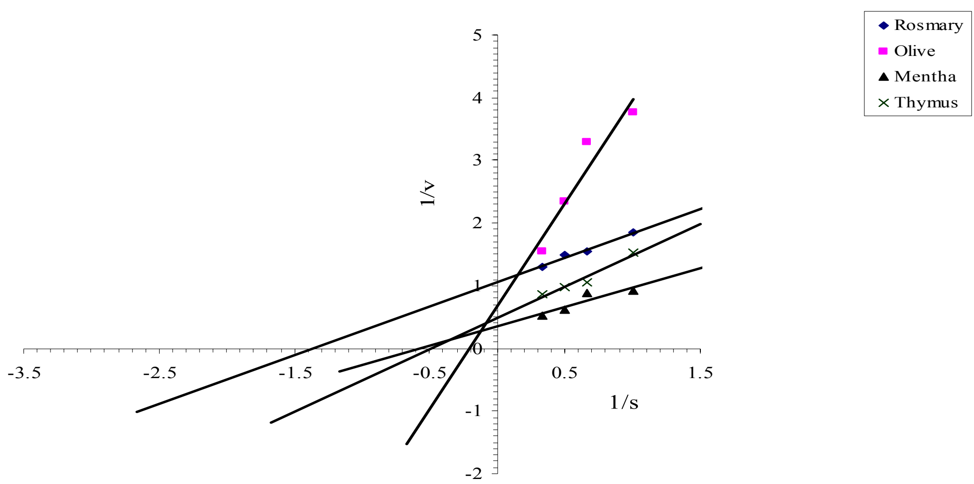


Figure (14)

Determination of K_m and V_{max} values for crude enzyme extract of the selected plants using egg albumin as substrate .

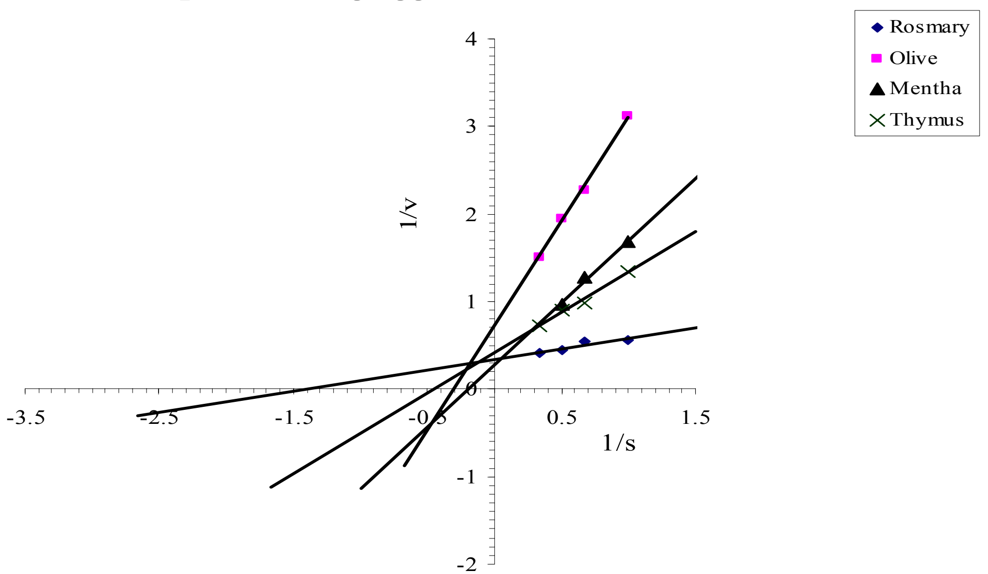


Figure (15)

Determination of K_m and V_{max} values for crude enzyme extract of the selected plants using casein as substrate.

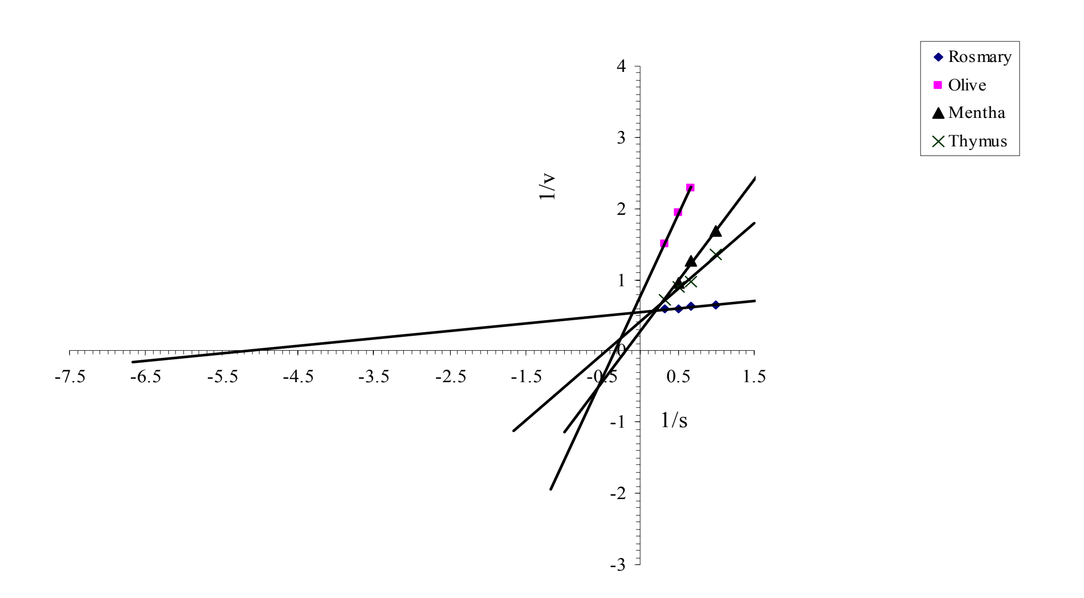


Figure (16)
Determination of K_m and V_{max} values for crude enzyme extract of the selected plants using albumin as substrate.

4.5 Substrate Specificity

Studying the kinetics of any enzyme is consider as a very important value in understanding the affinity and conditions suitable to obtain optimum activity particularly if these enzymes are studied to be used for industrial and biochemical applications. Proteases are one of the most groups of enzymes that play a role in many fields of industry.

According to the selected plants, three types of substrates have been used to study the enzymes substrate specificity. Casein, albumin and egg albumin were evaluated for their specificity and affinity towards the activity of proteases of *Rosmarinus officinalis, Mentha piperita, Olea europaea* and *Thymus capitatus.* Figure (17) illustrates the relative activity(%) of crude enzyme extracts (proteases) of the above plants at optimum pH and temperature. Casein has the highest relative activity (100%) for proteases of *Rosmarinus officinalis* followed by albumin(70%) and egg albumin with 30% relative activity. The least activity (20 to 30%) was seen for proteases of *Olea europaea*, on the other hand, intermediate relative activity (63%, 58% and 50%) of *Thymus capitatus* proteases were seen for the three substrates: casein, albumin and egg albumin, respectively. The activity is different according to the substrate specificity for the proteases of *Mentha piperita.* It was found that the maximum activity of proteases of *Mentha piperita* for the substrates albumin, egg albumin and casein was as follows: 100%, 79% and 43%, respectively. Our results demonstrate that casein has high substrate

specificity for the proteolytic activity of protease enzyme of *Rosmarinus officinalis, Olea europaea* and *Thymus capitatus.* The same results was found by Mahajan, (2012). They reported that casein can be used as a good substrate for many plant proteases such as *Carica papaya, Calotropis procera, Euphorbia nivulia* and *Ficus carica.*

According to the obtained data from Lineweaver-Burk plots, Substrate specificity (V_{max}/K_m) was calculated . Results in table (4) showed different activities towards different substrates. K_m value is a measure of affinity of the proteases for the substrate, the smaller the value the higher the affinity of the enzyme for the substrate while V_{max} shows the catalytic efficiency, the higher the V_{max} the higher the efficiency.

According to Palmer (1995), the best substrate for any enzyme depends on the strong binding of the substrate to the enzyme (low K_m) and high proteolytic activity (high V_{max}). By considering the ratio (V_{max}/K_m) (Table 4), it was observed that casein was the best substrate for proteases of *Rosmarinus officinalis* (4.64) followed by *Thymus capitatus* (1.813), *Olea europaea* (0.64) and *Mentha piperita* (0.4). On the other hand, egg albumin was the best substrate for proteases of *Mentha piperita* (1.65) followed by *Rosmarinus officinalis*(1.145) , *Thymus capitatus,* (1.0) and *Olea europaea* (0.24).

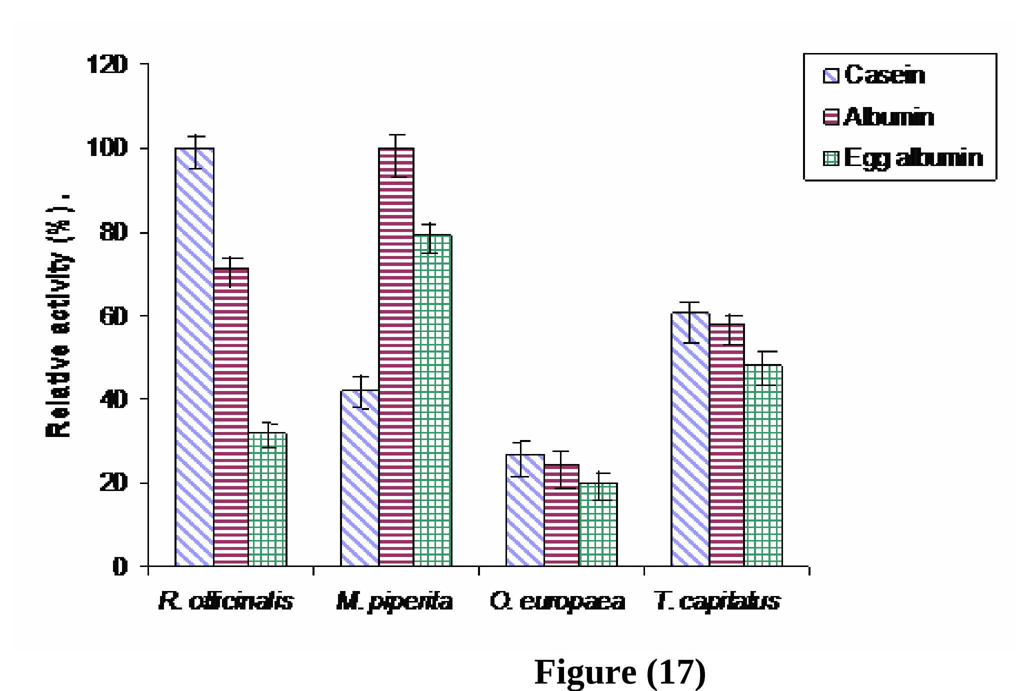


Figure (17)

Comparison between the relative activities (%) of the crude enzyme extracts (proteases) of the selected plants . Mean ± SD, n=3.

Table (4)
Comparison between the V_{max}, K_m and the ratio of V_{max}/K_m for the crude enzyme extracts (proteases) of the selected plants in means of casein, albumin and egg albumin as substrates .

Sample	Casein			Albumin			Egg albumin		
	V_{max} (U/mg)	K_m (mg/ml)	V_{max}/K_m	V_{max} (U/mg)	K_m (mg/ml)	V_{max}/K_m	V_{max} (U/mg)	K_m (mg/ml)	V_{max}/K_m
Mentha piperita	2.0	5.0	0.4	5.0	5.0	1.0	3.3	2.0	1.65
Rosmarinus officinalis	3.3	0.71	4.65	0.2	0.21	0.95	0.8	0.7	1.14
Thymus capitatus	2.9	1.6	1.812	2.5	1.6	1.56	2.0	2.0	1.0
Olea europaea	1.6	2.5	0.64	1.25	2.5	0.5	1.2	5.0	0.24

4.6 Conclusion

Plant green leaves of *Rosmarinus officinalis, Olea europaea, Mentha piperita* and *Thymus capitatus* were analyzed for protease activities. The comparative studies on the proteolytic activity profile of the crude enzyme extracts for the selected plants indicated that they belong to the acidic and neutral protease groups.

The pH and temperature studies, revealed that these proteases are potential endopeptidases with many properties such as optimum pH and optimum temperature. It was found that *Rosmarinus officinalis* and *Olea europaea* exhibiting optimum pH around 7.0, on the other hand, *Thymus capitatus* exhibiting two optimum pH around 3.0 and 6.5, while *Mentha piperita* exhibit two optimum pH around 3.0 and 9.0. Whereas, the optimum temperature range was found in between 35-45°C.

According to the results of K_m and V_{max}, it was illustrated that casein is the best specific substrate for proteases of *Rosmarinus officinalis* followed by *Thymus capitatus,* and *Olea europaea* and egg albumin was the best substrate for proteases of *Mentha piperita followed by Rosmarinus officinalis* and *Thymus capitatus.*

4.7 Recommendation

1- Further experiments are needed for purification and biochemical characterization of the proteases from these selected plants.
2- Results are encouraging to many useful application in industry and biotechnological industries.

References

Al-sherhri, MA., Mostafa ,S .(2004). Products form and properties of protease produced by Bacillus licheniformis. **Biological science** 7(9):1631-1635.

Al-Shehri, M.A., (2004). Production and some properties of protease produced by Bacillus licheniformis isolated from Tihamet Aseer, Saudi Arabia. **Pakistan J. Biol. Sci**., 7: 1631–5

Akhtaruzzaman, S. (2012). Role of thiol proteases in tissue injury.Am. J. Resp. Crit. **Care Med**. 150:S155–59

Barindra S, Debashish G, Malay S. and Joydeep M. (2006). Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma Proteobacterium isolated from the marine environment of the Sundarbans. **Process Biochem**. 41: 208–215.

Batkin, S., S.J. Taussig and J, Szekerczes .(1988). Modulation of pulmonary metastasis (Lewis lung carcinoma) by bromelain and extract of the pineapple stem (Ananas comosus). **Process Biochem**., 6: 233-234.

Beers, EP., Woffenden ,BJ, Zhao, C .(2000) Plant proteolytic enzymes:possible roles during programmed cell death. **Plant Mol Biol.,** 415-44:399

Bewley, JD. and Black, M,(1994). Seeds: Physiology of development and germination. **Ed. New York, London, Plenum Press.** 245-401.

Beynon, J. and J.S, Bond. (1989). Proteolytic enzymes, a practical approach. IRL Press, New York, NY.Bifidobacterium longum inhibits eukaryotic elastase like serine proteases. **J. Biol. Chem**. 281(25): 17246-52.

Capiralla, H., Hiroi, T., Hirokawa, T. and Maeda, S. (2002) .Purification and characterization of a hydrophobic amino acid-specific endopeptidase from alobacterium halobium S9 with potential application in debittering of protein hydrolysates. **Process Biochemistry.** 38: 571-579

Charles P, Devanathan V, Anbu P, Ponnuswamy M N, Kalaichelvan P T & Hur B K (2008). Purification , characterization and crystallization of an extracellular alkaline protease from Aspergillus nidulans HA-10, **J Basic Microbiol**, 48: 347-352.

Cheng, K., Lu, F. P., Li, M., Liu, L. L. and Liang, X. M. (2010). Purification and biochemical characterization of a serine alkaline protease TC4from a new isolated Bacillus alcalophilus TCCC11004 in detergent formulations. **African Journal of Biotechnology**. 9: 4942-4953.

Chouyyok, W., Wongmongkol, N., Siwarungson, N. and Prichnont, S. (2005). Extraction of alkaline protease using an aqueous two-phase system from cell free Bacillus subtilis TISTR 25 fermentation broth. **Process Biochem**. 40: 3514–3518.

Christeller, J.T. (2005). Evolutionary mechanisms acting on proteinase inhibitor variability. **FEBS Journal.** 272: 5710 – 5722.

Demuth, H. (1990). Enzyme Inhibition, **Journal of biological s**. 3- 249-278.

Domsalla, A.and Melzig, M.F. (2008). Peptidase inhibitors in the MEROPS database. **Biochimie**. 92: 1463-1483. Rawlings N.D.,

Dorman, H. J. D., A, Peltoketo., R, Hiltunen. and M. J, Tikkanen. (2003). Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. **Food Chem**. 83:255-262.

Erlacher, A., Sousa, F., Schroeder, M., Jus, S., Kokol, V., Cavaco-Paulo, A.and Guebitz, G. M. (2006). A new cuticle scale hydrolysing protease from *Beauveria brongniartii*.. **Biotechnol Lett**, 28:703–710.

Fahmy, A. S., Ali, A. A., and Mohamed, S. A. .(2004). Characterization of a cysteineprotease from wheat Triticum aestivum. **Bioresource Technology**. 91: 297-304.

Genelhu, M.S., Zanini, I.F., Veloso, A.M.D., Carneiro,M.T.P., Lopes and C.E. Salas .(1998). Use of a cysteine proteinase fromCarica candamarcensis as a protective agent during DNA extractionBrazilian **Journal of Medical and Biological Research** 31: 1129-1132.

Gruenwald, J., Brendler, T . and Jaenicke, C .(2004). PDR for Herbal Medicines, 3rd dition. M **Process Biochem**, pp.815-816.

Guimarães-Ferreira, J. and J, Miguéns. (2007). "Advances in cranioysinostosis research and management." **Adv Tech Scand Neurosurg**.

Gupta, R., Beg, Q. K., and Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. **Applied Microbiology and Biotechnology**. 59: 15-32.

Hajji, M., Kanoun, S,. Nasri ,M. and Gharshallah, N. (2007). Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1. **Proc Biochem**. 42: 791-797.

Haq, I., Mukhtar, Z.A. and Riaz, N. (2004). Protease biosynthesis by mutant strain of Penicillium griseoroseum and cheese formation. **Pakistan J. Biol. Sci**. 7: 1473–1476.

Ibrahim, h. r., yamada, m., matsushita, k., kobayashi, k and kato, a. (2012a). Enhanced bactericidal action of lysozyme to Escherichia coli by inserting a hydrophobic pentapeptide into its C terminus. **J. Biol. Chem**. 269: 5059-5063.

Ibrahim M. A., J. O. Olatominwa, A. B., Aliyu, M., Bashir, A. B. Sallau. (2012b). Partial Characterization of Protease from the Leaves of Jatropha curcas, **International Journal of Biology.** 79-85.

Ichishima,E., Yamaguchi,M., Yano,H., Yamagata,Y. and Hirano,K. (1991). C-Terminal peptidyl-L-proline hydrolase activity of Aspergillus acid carboxypeptidase Agric. **Biol. Chem**. 55, 2191–2193.

Ivanov, D., Emonet, C., Foata, F., Affolter, M., Delly, M., Fisseha, M., Blum-Sperisen, S., Kochhar, S. and Arigoni, F. (2006). A serpin from the gut bacterium Bifidobacterium longum inhibits eukaryotic elastase like serine proteases. **J. Biol. Chem**. 281(25): 17246-52.

Jakubowski, H.(2013).**Biochemistry Online: An Approach Based on Chemical Logic**. College of Saint Benedict , Saint John's University

Joanitti. GA, Freitas, SM. and Silva ,LP. (2006). Proteinaceous Protease Inhibitors: Structural Features and Multiple Functional Faces. **Current Enzyme Inhibition** 2(3): 199-217.

Johnvesly, B. and Naik, G. R. (2001). Pigeon pea waste as a novel, inexpensive, substrate for production of thermostable alkaline protease from thermoalkalophilic *Bacillus* spp. **Proc Biochem**, 37: 139-44.

Katunumaet *, 2003).* Cell-derived proteases and protease inhibitors asregulators of neurite outgrowth. **TINS** 11, 541-544

Kelly, M., (1996). "The Dynamics of Smithian growth . **Process Biochem**. 96:9.

Kessler RC, Walters EE, Forthofer MS .(1998). The social consequences of psychiatric disorders, III: Probability of marital stability. **Arch Gen Psychiatry.** 155:1092–1096.

Khorshidi K.J., Rezaean M., Zahedi Far M. and Mirhadi S.A.(2009). Effect of defaunating the rumen of sheep fed various diets on gas production *in vitro*. **J. Pajouhesh-Va-Sazandegi**. **73,** 30-35.

Kleef ,R,. Delohery, T., Boubjerg ,D .(2004). Selective modulation of cell adhesion molecules on lymphocytes by bromelain protease 5. **Pathobiology.** 1996;64:339–46.

Kotlova E K, Ivanova N M, Yusupova M P, Voyushina T L, Ivanushkina N E & Chestukhina G G (2007), Thiol-Dependent Serine Proteinase from Paecilomyces lilacinus: Purification and catalytic properties, **Biochem (Moscow**), 72: 117-123.

Kumar C.G. (2001)Purification and characterization of a thermostable alkaline protease from alkalophilic Bacillus **pumilus Volume 34, Issue ,** pages 13–17, January

La Valle J, Krinsky D, Hawkins E.(2000) Natural therapeutics pocket guide. **Hudson, Ohio**: Lexi- Comp;.

Lawrence, PK. and Koundal, KR. (2002). Plant protease inhibitors in control of phytophagous insects. **Electronic Journal of Biotechnology** 5(1): 93-109.

Lee-Huang, S., Zhang, L. and Huang, PL. (2003). Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. **Biochem Biophys Res Commun**. 307:1029-1037.

Leipner, J., Ilen, F., Saller, R.(2001). Therapy with proteolytic enzymes in rheumatic disorders. **Bio Drugs**.15:779–89.

Leary D, Vierros M, Hamon (2009). Marine genetic resources: a review of scientific and commercial interest. **J. Biol. Chem**.;33:183-94.

Liao, C.H. and D.E. McCallus. (1997). Biochemical and genetic characterization of an extracellular protease from Pseudomonas fluorescens CY091. **Appl.** Environ. Microbiol. 64:914-921.

Li, D, Yang, & Shen C Y (1997). Protease production by the thermophilic fungus Thermomyces lanuginosus, **Mycol Res**, 101: 18-22

Li, M.S., Y.P. Luo and Z.Y. (2007) : Heavy metal concentrations in soils and plant accumulation in a restored manganese mineland in Guangxi,South China. **Environ. Pollut**., 147, 168-175

Lotti ,T.(1993) Controlled clinical studies of bromeline in the treatment of urogenital inflammation. **Bio Drugs.** ;46:144–6.

Lowry, O.H., N.J., Rosebrough., A.L. ,Farr. and R.J., Randall. (1951). Protein measurement with the Folin phenol reagent. **J. Biol. Chem**. 193:265-275.

Mahajan, B (2012). Selective targeting of lysosomal cysteine proteases with radiolabeled electrophilic substrate analogs, **Chemistry & Biology.** (7):27–38.

Marchetti, S., Chiaba, C., Chiesa, F., Bandiera, A. and Pitotti, A. (1998). Isolation andcharacterization of two trypsins from the larval midgut of Spodoptera littoralis(Boisduval). **Insect Biochem Mol Biol**. 28: 449-458.

McKevitt, A., S. Bajaksouzian, J.D. Klinger, D.E. Woods. (1989). Purification and characterization of an extracellular protease from Pseudomonas cepacia. Appl. Environ. Microbiol. 57:771-778.

Melis, G. (1990). Clinical experience with metoxybutropate vs bromelain in the treatment of female pelvic inflammation**. Minerva Ginecol.** 42:309–12.

Mello, J. C. C. B., Gomes, E. G., Angulo-Meza, L. and Biondi Neto, L. (2008). **Cross evaluation using weight restrictions in unitary input dea models: Theoretical aspects and application to Olympic games ranking. WSEAS Transactions on Systems,** Forthcoming**.**

Moallaei, H., Zaini, F., Larcher, G., Beucher, B. and Jean-Philippe Bouchara,J. P. (2006). **Mycopathologia**, 161: 369–375.

Monard, D. (1988). Cell-derived proteases and protease inhibitors asregulators of neurite outgrowth. **TINS** 11, 541-544

Munilla-Morán, R. and Saborido-Ray, F. (1996). Digestive enzymes in marine species. I. Proteinase activities in gut from redfish)Sebastesmentella), sea

bream (Sparusaurata) and turbot (Scophthalmusmaximus). Comparative **Biochemistry and Physiology**. B: 395-402.

Mykles, D. L.(2001) . Methods in Cell Biology, .against *Tetranychus cinnabarinus*. **J. Beijing** Univ Agric 24: 17-21

Ogino, H., Watanabe, F., Yamada, M., Nakagawa, S., Hirose, T., Noguchi, A., Yasuda, M. and Ishikawa, H. (1999). **J Biosci Bioeng, 87: 61–68.**

Olsen SF, Sorensen JD, Secher NJ, Hedegaard M, Henriksen TB, Hansen HS, et al. (1992). Randomised controlled trial of effect of fish-oil supplementation on **pregnancy duration.Lancet** 339:1003–1007

Otsuki N, Dang NH, Kumagai E, Kondo A, Iwata S, Morimoto C.(2010). Aqueous extract of Carica papaya leaves exhibits anti-tumor activity and immunomodulatory effects. **J. Ethnopharmacol**.127:760–7.

Padul M.V., Tak R.D. and Kachole M.S. (2012). Protease inhibitor (PI) mediated defense in leaves and flowers of pigeonpea (protease inhibitor mediated defense in pigeonpea). **Plant Physiol. Biochem.** 52: 77-82.

Palmer JT, Rasnick D, Klaus JL, Bromme D, (1995) Vinyl sulfones as mechanism-based cysteine protease inhibitors. **J Med Chem** 38:3193±3196.

Paranthaman ,R., Alagusundaram, K. and Indhumathi, J. (2009). Production of protease from rice mill wastes by Aspergillus niger in solid state fermentation. **World J of Agri Sci**. 5(3): 308-312

Pardo, MF., Caffini ,NO., Lopez, L.M.I.and Hieronymain, I. (2000). a new cysteine peptidase isolated from unripe fruits of *Bromelia hieronymi* Mez (Bromeliaceae). **J. Prot Chem**. 22:127–34.

Pardo, MF., Lopez, M.I., Canals, F., Zviles, F.X., Natalucci ,C.L. and Caffini, N.(2000). Purification of balansain I, an endopeptidase from unripe fruits of *Bromelia balansae* Mez (Bromeliaceae). **J. Agric Food Chem**. 48:3795–800.

Paris, A. (1993). Inhibitory effect of carnosolic acid on HIV-1 protease in cell-free assays . **J Nat Prod**. 56 : 1426-1430 .

Patil, U K. (2010)., **Studies on novel thermostable and solvent-tolerant alkaline protease from bacteria**, Ph.D. Thesis, North Maharashtra University, Jalgaon (Maharashtra State), India.

Polgar, L. (1989). Mechanisms of protease action. CRC Press, **Inc., Boca Raton, Florida**. Pgs. 43-76.

Powers, J. C., Asgian, J. L., Ekici, O. D., James, K. E. (2002). Protease Action. **Chemical reviews**(Washington, DC, United States), 102, 4639-4750.

Rasooli, I., M.B. Rezaei and A. Allameh. (2006). Ultra structural studies on antimicrobial efficacy of thyme essential oils on listeria monocytogenes. **International Journal Infectious Diseases**. 10: 236-241.

Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. **Microbiol Mol Biol Rev**, 62: 597-635.

Rattray, F.P., Bockelmann, W. & Fox, P.F. (1995) Purification and characterization of an extracellular proteinase fromBrevibacterium linens ATCC 9174. **Applied Environmental Microbiology** 61 , 3454–3456.

Rawlings ND, Barrett AJ, Bateman A.(2010) .MEROPS: the peptidase database. **Nucleic Acids Res**;38:D227–33.

Rawlings, N. D., Morton, F. R., Barrett. (2006). Nucleic Acids **A. J. Research**, 34, D270-D272.

Rawlings, N.D., Tolle, D.P. and Barrett, A.J. (2004). Evolutionary families of peptidase inhibitors, **J. Biochem**. 378: 705-716.

Ren, JJ., Shi ,G., Wang ,J.W., Zheng ,Y., Wang, Y.N. (2009) Contact toxicity of crude extracts from thirty-one acaricidal plants in Northeastern China against *Tetranychus cinnabarinus*. **J. Beijing Univ Agric**. 24: 17-21

Rozs, M. (2001). Ermentation characteristics and secretion of proteases of new keratinolytic strain of *Bacillus licheniformis*. **Biotechnology Letters** . Vol. 23 Issue 23, p1925-1929. 5p

Ryan, C.A. (1990). Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. **Annu. Rev. Phytopathol.** 28: 425-449.

Salas CE, Gomes MTR, Hernandez M, Lopes MTP. (2008). Plant cysteine proteinases: evaluation of the pharmacological activity. **Phytochemistry**;69:2263–9.

Salas C.L., Fernándes, M.M., and Ana, M.M. de la C. (2005), Topical chemotherapy for the treatment of burns, **Revista De. Enfermería** (Barcelona, Spain), 28: 67-70

Samova, L.I., Shode ,FO.and Mipando, M. 2004. Cardiotonic and antidysrhythmic effects of leanolic and ursolic acids methyl maslinate and uvaol. **Phytomedecine**. 11(2-3):121–129.

Sandhya, C., Sumantha, A,. Pandey, C,. Webb, C.R. and Soccol, C.(2004). Proteases In: Enzyme Technology, Asiatech Publishers Inc., **New Delhi, India** pp. 312–325.

Sasaki, K., K. Wada., Y. Tanaka., T, Yoshimura. and K, Matuoka. (2005). Thyme (Thmus Vulgaris L.) Leaves and its constituents increase the activities of xenobiotic-metabolizing enzyme in mouse liver. **J. Med. Food**. 8: 184-189.

Schaller, G.E. and J.J, Kieber. (2004). **Ethylene in The Arabidopsis Book**

Sexton, M.M., A.L. Jones, W. Chaowagul, and D.E. Woods. (1994). Purification and characterization of a protease from Pseudomonas pseudomallei. Can. **J. Microbiol.** 40:903-910. Ota, N.; Agard, D. A. Protein Sci. 2001, 10, 1403-141

Shasany, K., Suman, P .S., Sunita, D. and Sushil, K. (2000). Positive correlation between menthol content and in vitro menthol tolerance in *Mentha arvensis L. cultivars*. **(CIMAP). Lucknow**. 226- 015

Silva, S. V. and Malcata, F. X. (2005). Studies pertaining to coagulant and proteolytic activities of plant proteases from Cynara cardunculus. **Food Chemistry**. 89, 9-26.

Sjodahl, J., Emmer, A., Vincent, J. and Roeraade, J. (2002).Characterization of proteinases from Antarctic krill (*Euphausia superba).* **Protein Expression and Purification.** 26:16-153.

Smith, L. S. and Halver, J. E. (2005). Digestive functions in teleost fishes. **In: Fish Nutrition.** London, pp. 31-421

Solomon, E. P., Berg, L. R., Martin, D. W. and Villee, C. (1996). **In: Biology**. Saunders College Publishing, Philadelphia, pp. 982-993.

Tang J., Li H.L., Shen Y.H., Jin H. Z., Yan S.K., Liu R.H. and Zhang W.D. (2008). Antitumor activity of extracts and compounds from the rhizomes of *Veratrum dahuricum*. **Phytother. Res.** 22: 1093-96.

Targoni, O.S., Tary-Lehmann M. and Lehmann‹ P.V. (1999). Prevention of murine EAE by oral hydrotic enzyme treatment**. J. Autoimmune**. 12: 191-198

Tatyana, A.V., Tatyana A.R., Galina V.K. and Vladimr V.M.(1998). Kunitz type protease inhibitors from intact and Phytophthora infected potato tubers. **FEBS Lett.** 426: 131–134.

Tunga, R., Shrivastava, B. and Banerjee, R. (2003).Purification and characterization of a protease from solid state cultures of Aspergillus parasiticus. **Process Biochemistry**. 38: 1553-1558.

Turk, V. and Bode, W. (1999). The cystatins: Protein inhibitors of cysteineproteinases. **FEBS Lett**. 285: 213–219.

Turk B, Turk V, Turk D. (1997) .Structural and functional aspects of papain-like cysteine proteases and their protein inhibitors. **Biol Chem**;378:141–50.

Uhlig, H. (1998). **Industrial enzymes and their applications**. New York : John Wiley and Sons. pp.37-202.

Ustariz FJ, Laca A, Garcia LA, Diaz M, (2004).Fermentation conditions increasing protease production by Serratiamarcescens in fresh whey. **Rev. Tec. Fac.** Ing. Univ., 31: 79–89.

Vanndita, B., Parth, K., Prasidhi ,T. and Kameshwar, S. YVR. (2013). Enzymatic determination of Catechol oxidase and Protease from fruits (orange, apple) and vegetables (carrot, tomato). IOSR **Journal of Pharmacy and Biological Sciences** . 5(5):29-35.

Voet, D. and Voet G. (2004).**Biochemistry(3rd ed).** John wiley and sons Inc. USA.leaf. Planta Medica. 1991;57(5):417–419

Wang ,L. and Wang YJ .(2004). Rice starch isolation by neutral protease and high-intensity ultrasound**, J. Cereal Sci**. 39 : 296-291
Zarzuelo , A. (1991). Vasodilator effect of olive leaf. **Planta Med**., 57: 417- 419.

Zibaee, A. & Bandani, A. R. (2009). Purification and characterization of the cuticle-degrading protease produced by the entomopathogenic fungus, Beauveria bassiana in the presence of Sunn pest, Eurygaster integriceps (Hemiptera: Scutelleridae) cuticle. **Biocontrol Science & Technology** 19, 797-808.

Appendix I
Instruments, Equipments and Materials

Instruments and equipments :

1. Analytical balance. OHAUS . USA.
2. spectrophotometer UV/ Vis Biotech Engineering Management co. LTD, UK.

Chemicals and Materials :

1- Sodium-potassium Tartrate . Sigma-Aldrich, Germany.
2- Cuppric sulfate penta hydrate. Sigma-Aldrich, Germany.
3- Soduim carbonate. BDH .GPR. England .
4- Phenol reagents (Folin - Ciocalteu) s. d. fine- CHEM Ltd.
5- Sodium phosphate-3-hydrate , 99-101 % max.0.00002% al, extra pure,DAB,PH.EUR,B.P.,PH. Franc.
6- Sodium acetate-3-hydrate , 99-101 % max.0.00002% al, extra pure,DAB,PH.EUR,B.P.,PH. Franc.
7- Acetic Acid Glacial , ACS Reagent Min. 99.7 %. NenTech Ltd., UK.
8- Trichloroacetic acid. TEDIA Company, Inc., USA.
9- Casein , Sigma-Aldrich, Germany.
10- Albumin , Sigma-Aldrich, Germany.
11- Egg albumin , BDH .GPR. England .

Appendix II
Standard Curve for total protein concentration

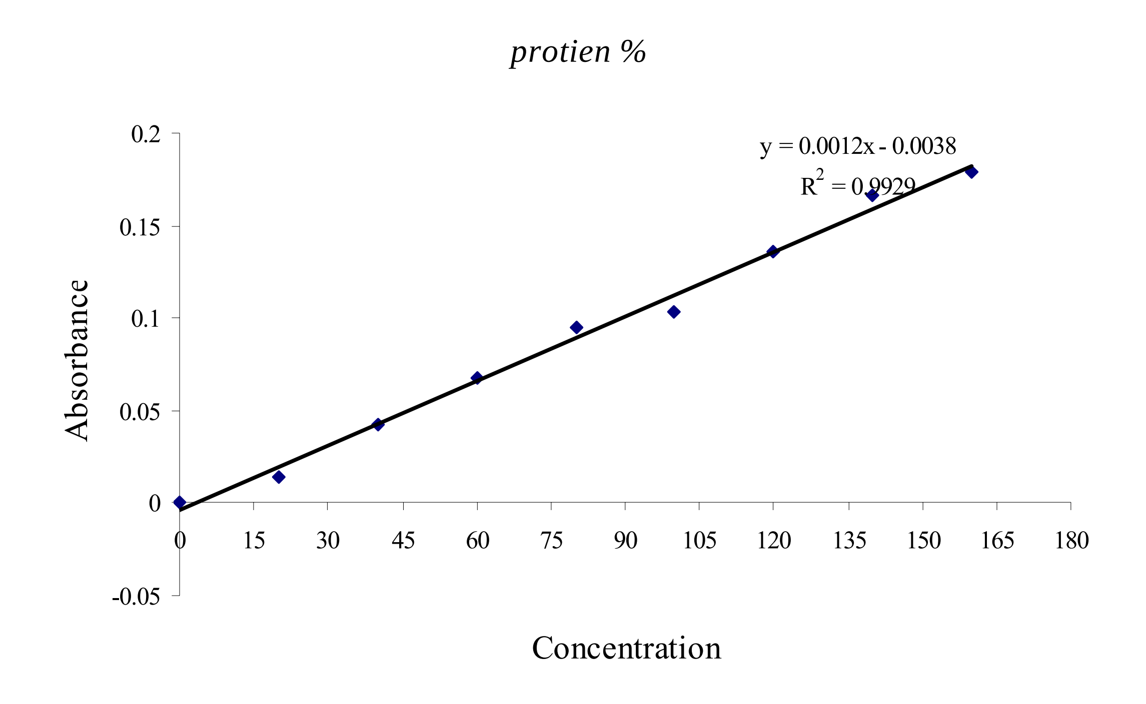


Figure (1)
Standard curve for total protein concentration